OCCURRENCE OF PATHOGENIC STRAINS OF YERSINIA ENTEROCOLITICA IN PIGS AND THEIR ANTIMICROBIAL RESISTANCE

JANA SIMONOVA, GABRIELA BORILOVA, AND IVA STEINHAUSEROVA

Department of Meat Hygiene and Technology, Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Science, 612 42 Brno, Czech Republic
pokorna.jm@post.cz gborilova@vfu.cz

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Abstract

Between 2005 and 2007, the incidence of Y. enterocolitica in pigs and particularly of its human pathogenic serotypes O:3 and O:9, was monitored. In 1 706 samples collected from the tongue, skin surface, skin surface near the rectum, rectal content, and tonsils, 76 pathogenic strains of Y. enterocolitica were identified. Y. enterocolitica was found most frequently on the tonsils (7.5%) and in rectal content (7.4%), and less frequently on the tongue (5.1%), skin surface (2.8%), and skin surface near the rectum (1.0%). Eighty-eight per cent of the strains belonged to the serotype O:3, one strain isolated from skin surface was identified as serotype O:5, and 8 strains were not specified. Using the reference agar dilution method, susceptibility to six antimicrobial agents (tetracycline, nalidixic acid, chloramphenicol, erythromycin, ciprofloxacin, and gentamicin) was tested. The strains showed high MIC levels to erythromycin and tetracycline. On the other hand, they were susceptible to the nalidixic acid, gentamicin, chloramphenicol, and ciprofloxacin. The highest susceptibility was found to ciprofloxacin.

Key words: pigs, Yersinia enterocolitica, pathogenic serotypes, antibiotic resistance.

Y. enterocolitica is divided into 60 serotypes, of which only 11 serotypes are important with regard to food-borne diseases. The most important of them are serotypes O:3, O:9, O:5,27, and O:8 (23).

The transfer of pathogenic strains of Y. enterocolitica to humans occurs primarily during food consumption if hygienic rules are not kept during food processing and/or storage. Even chilled food (meat, milk, and vegetables) may pose a risk of infection because Y. enterocolitica strains can survive at low temperatures (-4°C) (10, 21).

Y. enterocolitica is responsible for intestinal infections of different levels of severity. The most frequent is enteritis, enterocolitis, and mesenteric lymphadenitis, as well as for parenteral infections, which may even progress to septicaemia especially in children and patients with impaired immunity (5). The countries with the highest occurrence of yersiniosis include Germany and the Scandinavian countries. In 2005, Germany, Finland, and Sweden reported 5 624, 638, and 744 cases of yersiniosis, respectively (EFSA 2005). The number of yersiniosis infections in the Czech Republic has also been increasing slightly in recent years. While in 2000, 231 cases were registered, and in 2005, there were 498 cases (EFSA 2004 and 2005).

The administration of antimicrobial agents for the treatment of bacterial infections in both veterinary and human medicine poses a potential risk because it leads to the selection of strains resistant to antibiotics. A high incidence of resistant bacteria has particularly been reported from developing countries, where antibiotics are freely available and their use is not subjected to any regulation (16). The main reason for the increase in resistant bacteria population is apparently in the application of antibiotics as prophylactics and growth stimulants in animals (1, 22). For that reason, the use of antibiotics was gradually restricted, and in 2006, their use as growth stimulants was completely banned in the EU.

In the Czech Republic, systematic monitoring neither of the occurrence of pathogenic strains of Y.
**Material and Methods**

**Sample collection, cultivation, isolation, and identification of Y. enterocolitica strains.** Between 2005 and 2007, 1,706 samples from 13 different farms in the Czech Republic were collected from pigs at abattoirs (Czech Large White x Landrace x Duroc). The samples were obtained as smears (Amies Agar Gel Medium, Copan Italia S.p.A), from rectal content, and from bedding and evisceration. The smear samples were taken after bleeding, samples from the tongue, tonsils, rectal content, and skin surface in the rectum region after bleeding and evisceration. The smear samples were placed to the transport Amies Agar Gel Medium and taken to the laboratory for immediate processing. *Y. enterocolitica* strains cultivation was performed in accordance with the standard CSN EN ISO10273. The isolates were propagated in ITC Broth Base (M843, HiMedia India) for 48 h at 24°C, and then cultivated on Selective Agar base (CIN) (M1220, HiMedia India) for 24 h at 30°C. Suspected colonies from the 24 h culture on CIN agar were collected and used for DNA isolation.

**DNA extraction.** Bacterial DNA was prepared by the phenol-chloroform method as described by Sambrook et al. (20). Briefly, colonies identified as *Y. enterocolitica* were suspended in Tris-HCl-EDTA buffer, pH 8.0, frozen, thawed, and subsequently lysed by incubation with proteinase K at 55°C overnight. The DNA released was extracted twice with an equal volume of phenol:chloroform:isoamylalcohol (Serva, Germany) and once with chloroform. DNA concentrated by ethanol precipitation was dissolved in 40 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at -20°C.

**Polymerase chain reaction.** The strains isolated were identified by the PCR method. For the detection of *Yersinia* genus strains, the PCR method based on the amplification of the specific DNA sequence of the 16S rRNA gene was used (2). Pathogenic strains of *Y. enterocolitica* were detected on the basis of the presence of the *ail* gene (23). Pathogenic strains of *Y. enterocolitica* serotype O:3 were identified by the PCR method based on the *rfbC* gene detection (24).

**PCR conditions.** The reaction mixture (12.5 µl) contained 1 µl of DNA template, 4.2 µl of H₂O, 6.3 µl of PCR-Mix (PP MASTER MIX, Top-Bio s.r.o., CR), and 0.5 µl of each primer (0.01 nM.µl⁻¹). PCR products (300 bp, 425 bp, and 405 bp) were analysed on 3% agarose gel (Serva, Germany) run at 120 V in a Tris-HBO₃-EDTA buffer, pH 8.3. The DNA fragments were stained with ethidium bromide.

**Serotyping.** Serotypes O:5, O:8, and O:9 of the isolated pathogenic strains were identified by slide agglutination using commercially available antisera (Itest plus, CR).

**Minimum inhibitory concentration (MIC) detection.** Antibiotic susceptibility/resistance was tested by the agar dilution method in accordance with the CLSI guideline (7). The following antibiotics used: tetracycline (TET), nalidixic acid (NAL), chloramphenicol (CMP), erythromycin (ERY), gentamicin (GEN) (all Sigma-Aldrich, USA), and ciprofloxacin (CIP) (KRKA, Poland). The MIC determination was carried out on Mueller–Hinton agar (CM0337, Oxoid UK) supplemented with 5% sheep’s blood and with the relevant dilution of an antimicrobial drug. The dilutions ranged from 0.25 to 128 µg/mL except for ciprofloxacin and gentamicin (0.063 to 32 µg/mL). Bacterial suspension (1 x 10⁷/mL) was prepared from 24-h *Y. enterocolitica* culture in Brain Heart Infusion (Oxoid, UK). Approximately, 2 x 10⁶ bacteria/mL were put on antibiotic–containing Mueller–Hinton agar and incubated for 24 h at 30°C. The MIC was defined as the lowest concentration that produced complete inhibition of growth of *Y. enterocolitica* strains. Susceptibility category determination was performed complete inhibition of growth of *Y. enterocolitica* strains. Susceptibility categorisation was determined according to the CLSI guideline (6), the protocol: MIC Interpretative Standards (µg/mL) for Enterobacteriaceae. Isolates resistant to four or more antibiotics were considered multi-resistant. The reference strains of Campylobacter jejuni subsp. jejuni ATCC 33560 (CCM, CZ) and *Y. enterocolitica* CCM 5671 were used as control strains.

**Results and Discussion**

Between 2005 and 2007, a total of 1,706 samples were collected from pigs in abattoirs. In all the samples collected, 76 (4.5%) pathogenic strains of *Y. enterocolitica* were identified. A list of *Y. enterocolitica* strains isolated from the tongue, skin surface, and skin surface near the rectum, rectal content, and tonsils is given in Table 1.

*Y. enterocolitica* was found most frequently on swine tonsils (7.5%) and in rectal content (7.4%), and rarely on the tongue (5.1%), skin surface (2.8%), and skin surface in the rectal region (1.0%). Eighty-eight per cent of *Y. enterocolitica* strains isolated belonged to the serotype O:3, and one strain isolated from skin surface was identified as serotype O:5.
Table 1
Occurrence of pathogenic strains of *Y. enterocolitica* in pigs

<table>
<thead>
<tr>
<th>Source</th>
<th>No. of samples</th>
<th>No. of pathogenic strains</th>
<th>O:3</th>
<th>O:5</th>
<th>unidentified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin surface</td>
<td>394</td>
<td>11 (2.8%)</td>
<td>10</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Tongue</td>
<td>296</td>
<td>15 (5.1%)</td>
<td>15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tonsils</td>
<td>439</td>
<td>33 (7.5%)</td>
<td>30</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Skin surface in the rectal region</td>
<td>402</td>
<td>4 (1.0%)</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Rectal content</td>
<td>175</td>
<td>13 (7.4%)</td>
<td>8</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>1 706</td>
<td>76 (4.5%)</td>
<td>67</td>
<td>1</td>
<td>8</td>
</tr>
</tbody>
</table>

Table 2
Antibiotic susceptibility of *Y. enterocolitica*

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Number of strains with MIC (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;0.063</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>2</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>3</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>72</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>2</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>5</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>2</td>
</tr>
</tbody>
</table>

Serotypes of eight strains were not identified because of their negative reactions for O:5, O:8 and O:9 serotypes, and negative PCR reaction for the identification of *Y. enterocolitica* serotype O:3. *Y. enterocolitica* in pigs is harboured mainly in the oral cavity and in the intestinal tract (15). The most contaminated regions in our study were the tonsils, tongue, and the rectal content. The occurrence of pathogenic strains of *Y. enterocolitica* in pigs has been reported in numerous studies. Bonardi et al. (4) reported the presence of *Y. enterocolitica* in the intestinal content and on the tonsils. The presence of *Y. enterocolitica* in intestinal content was found in 4%. Almost all the strains belonged to the serotype O:3, one to the serotype O:9, and one strain was not identified. *Y. enterocolitica* was present in 14.7% of tonsil samples. Out of the strains found, 86% were of serotype O:3 and the remaining 14% were not identified. Very frequent detection of *Y. enterocolitica* strains in pigs (38.4%, 3.8%, and 0.3% on the tonsils, lymphatic nodes, and body surfaces, respectively) were also reported by Gürtler et al. (12). Almost all strains belonged to serotype O:3, and only one strain to serotype O:9. Johannessen et al. (13) monitoring the occurrence of pathogenic strains of *Y. enterocolitica* in pigs from five different slaughter houses, found in 15.2% of cases the pathogenic strains of *Y. enterocolitica* O:3.

Different pathogenic serotypes of *Y. enterocolitica* occur locally in specific regions. In Europe and Japan, the prevalent ones are the serotypes O:3 and O:9, while in the USA the most frequently ones that occur are the serotypes O:4, O:8, O:13a/b, O:18, O:20, and O:21. The only pathogenic serotype that occurs worldwide is the O:5, 27 (23). The 76 strains of *Y. enterocolitica* isolated from pigs were assayed for antimicrobial susceptibility/resistance by the reference agar dilution method. Susceptibility to six antimicrobial agents is shown in Table 2. All of the strains investigated were susceptible to gentamicin. The MIC of 85% of the isolates ranged from 0.5 mg/L to 2 mg/L, and none of them was detected as moderately susceptible or resistant. Similar results have also been reported in studies from Switzerland and Austria (3, 17). The authors found that none of isolates was resistant to gentamicin and (90 % - 100%) of isolates were susceptible to streptomycin, too.

**Susceptibility of *Y. enterocolitica* isolates of the serotype O:3 to tetracyclin was confirmed in 80.26% (MIC 1-4 mg/mL), moderate susceptibility was detected in 6.58% cases, and 13.16% of the isolates were TET-resistant. Similar results have been reported by Baumgartner et al. (3), who found the incidence of resistant *Y. enterocolitica* isolates in pigs at the 5%
level. Lower incidence of resistant strains may have been caused by the fact that, although tetracyclines have been suggested as an alternative in the treatment of clinical gastritis, they are rarely used in practice.

Although ciprofloxacin is an antimicrobial agent of high therapeutic importance, all the isolated strains of Y. enterocolitica serotype O:3 were CIP–susceptible with range of MIC <0.063-0.125 mg/L, and the susceptibility of 95% of strains were below the limit concentration tested of 0.063 mg/L. In 89% of the isolates, nalidixic acid MIC ranged from 1 mg/L to 8 mg/L. Only three (3.95%) isolates of Y. enterocolitica serotype O:3 were NAL–resistant. High (fluoro)quinolone susceptibility level of Y. enterocolitica strains isolated from pigs was reported also in other studies. Funk et al. (9) reported 100% of Y. enterocolitica strains to be susceptible to ciprofloxacin and Kwaga et al. (14) reported 100% of Y. enterocolitica strains to be susceptible to ciprofloxacin. The same results have also been reported by Baumgartner et al. (3), who detected 100% strains of Y. enterocolitica to be susceptible to ciprofloxacin.

High-level resistance against the macrolides, in particular erythromycin, was proved. All the strains of Y. enterocolitica serotype O:3 were found to be within the MIC range of 16–128 mg/L. A total of 31 isolates (40.8%) showed a high-level of ERY-resistance with MIC exceeding 128 mg/L. High-level resistance to macrolides (especially to erythromycin 95%–100%), tilimicosin, and tylosin was also reported in two other studies (9, 14). High resistance against this group of antibiotics can probably be traced to their use as growth stimulators (9, 14). High resistance against this group of antibiotics can probably be traced to their use as growth stimulators (9, 14). High resistance against this group of antibiotics can probably be traced to their use as growth stimulators (9, 14). High resistance against this group of antibiotics can probably be traced to their use as growth stimulators (9, 14). High resistance against this group of antibiotics can probably be traced to their use as growth stimulators (9, 14). High resistance against this group of antibiotics can probably be traced to their use as growth stimulators (9, 14).

The susceptibility to chloramphenicol (amphenicols) was confirmed in 67.11% of the examined isolates of Y. enterocolitica (28.95% of cases showed moderate susceptibility). Only three (3.95%) strains were CMP–resistant. The same results were reported by Baumgartner et al. (3).

The results of this study indicated that strains of Y. enterocolitica isolated from pigs showed a high degree of susceptibility to aminoglycosides (GEN), quinolones and fluoroquinolones (NAL, CIP), and amphenicols (CMP). The resistance was demonstrated only in the case of two groups of antibiotics: tetracyclines (TET, 13%) and macrolides (ERY, 100%). Multiresistance to the antimicrobial agents tested was not found in any of the Y. enterocolitica isolates. Because the strains that cause human enteritis may frequently come from farm animals directly or via contaminated food, it is important that the antibiotic resistance of the strains should be constantly tested. The use of antibiotics for the therapy of yersiniosis in animals will be thus improved.

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